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13. ABSTRACT *(Maximum 200 Words)*

The purpose of these studies was 1) to evaluate the interactions between Wortmannin-mediated inhibition of the Akt/PKB pathway and sphingolipid-induced apoptosis in a panel of human breast adenocarcinoma cell lines with basal or over-expression of HER-2/neu, and 2) to translate the formulation and toxicity studies conducted in the first year to initial proof-of-principle studies in nude mouse/human HER-2/neu over-expressing breast adenocarcinoma models. Investigations pertinent to Aims 1 (apoptosis) and 5 (efficacy studies) were the main focus. Progress in Aim 1 has been acceptable and studies are continuing in a model with low/basal expression and transfectants with high HER-2/neu expression. Our results indicate that inhibition of the Akt/PKB pathway enhances the apoptotic response to sphingolipids. Progress pertinent to Aim 5 has been stymied until very recently due to the lack of reproducible in vivo tumorigenic behavior of the MDA-361 HER-2/neu over-expressing human breast adenocarcinoma model. We believe that this can be linked to sub-optimal in vitro culture conditions which either select for variants or otherwise compromise subsequent tumorigenicity. With this obstacle apparently successfully overcome, we expect satisfactory progress on the remaining Tasks.

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Introduction

The purpose of these studies was 1) to evaluate the interactions between Wortmannin-mediated inhibition of the Akt/PKB pathway and sphingolipid-induced apoptosis in human breast adenocarcinoma cell lines with basal or over-expression of HER-2/neu, and 2) to translate the formulation and toxicity studies conducted in the first year to intial proof-of-principle studies in nude mouse/human HER-2/neu over-expressing breast adenocarcinoma models. Investigations pertinent to Aims 1 (apoptosis) and 5 (efficacy studies) were the main focus. Progress in the former Aim has been acceptable and studies are continuing. Progress pertinent to the latter Aim has been stymied until very recently due to the lack of reproducible in vivo tumorigenic behavior of the MDA-361 HER-2/neu over-expressing human breast adenocarcinoma model.

Body

Task 1

We have essentially completed studies characterizing the apoptotic responses of HER-2/neu basal-expressing (MDA-MB-231, MDA-MB-435, MDA-MB-468, and MCF-7) and high-expressing (BT-474, MDA-MB-453, SK-BR-3 and AU-465) human breast adenocarcinoma cell lines to C_6-ceramide, sphingosine and dimethyl-sphingosine, with and without Wortmannin. In general, these studies support the hypothesis that Wortmannin-mediated inhibition of the Akt/PKB anti-apoptotic pathway enhances the apoptotic response to these lipids.

In light of these results and based on new developments, we have decided to extend these studies to evaluation of this interaction(s) in a single, Her-2/neu basal-expressing host cell background, MDA-MB-435. This parental cell has been successfully transfected with Her-2/neu by a Faculty colleague, Dr. Dihua Yu, and three high-expressing clones have been selected. This approach offers certain advantages compared to selection of existing cell lines with presumably numerous genetic differences based on differential Her-2/neu expression. These studies will employ similar approaches to those previously used in the completed studies.

In addition, rather than simply using Wortmannin to inhibit the Akt/PKB pathway, we will characterize the functional status of this pathway in control and Wortmannin-treated cells. This will allow more definitive attribution of the effects of Wortmannin sensitization to sphingolipid-induced apoptosis to this signalling pathway.

Task 2

Studies herein were pending conclusive results from Task 1. Since these indeed now appear positive so far, we will proceed as planned with these studies.

Task 3

These formulation studies will now proceed given the positive results from Task 1 as described above.

Task 4

Long-circulating (PEG-containing) liposomes are pending formulation after completion of Aim 3. No difficulties in accomplishing this formulation are anticipated.

Tasks 5 and 6

In the first year, studies in nude mice progressed which indicated that a multiple-dose MTD of 4.0 mg was a more accurate figure than the 0.5-1.5 mg previously suggested by the literature and by our preliminary studies. These studies are now near completion with modest dose-escalation planned, again conducted in nude mice. These are important studies, since the apoptotic responses to sphingolipids appear to follow steep dose-response relationships, and anti-tumor effects may be missed in vivo with sub-MTD dosing. Studies in BALB/c mice will likely not be conducted, as our original intent, driven by cost considerations, was to first estimate MTDs in normal mice prior to nude mouse studies.

Task 7

Task 7, using long-circulating (PEG-containing) liposomes will be narrowed, for the reasons cited above, to nude mouse studies, foregoing studies in BALB/c mice.

Task 8

This task has taken most of the attention and effort this year, and until recently, has caused by far the most difficulty. We did not fully appreciate that there were limited options in terms of Her-2/neu over-expressing human breast adenocarcinoma models with predictable and characterized in vivo growth, particularly at the preferred orthotopic site.

In the first efficacy study with the HER-2/neu over-expressing MDA-MB-361 cell line, we failed to observe tumor outgrowth, even after several months. In this and in subsequent experiments described below, except for the most recent one, the 361 cells were cultured in bicarbonate-based DME'F12 tissue culture medium. In the second experiment, we used two HER-2/neu over-expressing models: 361 and SK-BR-3, which we obtained from Co-Investigator, Dr. Mien-Chie Hung. With the latter, implantation with Matrigel has been reported to enhance the efficacy of implantation. The SK-BR-3 model has the apparent disadvantage that initially growing tumors spontaneously regress; however, we felt that it would still have some merit in evaluating anti-tumor effects if treatment were initiated sufficiently early and an endpoint prior to the onset of spontaneous regression in controls was

employed. Unfortunately, again no tumor outgrowth was observed in either mode, so the treatment protocol was abandoned.

Next, we recognized that one possible cause for these failures and a source of variability could be the status of the inoculated tumor cells as well as laboratory-to-laboratory derivation of subclones with different tumorigenicity. Therefore, we purchased MDA-MB-361 cells from ATCC and cultured them strictly according to their guidelines, including the use of Leibowitz medium (non-CO_2/bicarbonate-based) and the use of serum specifically screened/recommended by and purchased from ATCC. These cells were successfully passaged several times at 1:2 or 1:4 and expanded 10-20-fold from the original 1 x 10^7 cells. During these passages, they retained their original morphology and growth pattern, which was typified by closely-packed islands of cells.

We first injected sentinel mice with these 361 cells to verify and establish the kinetics of tumor outgrowth. This outgrowth and monitoring is underway.

These cells were subsequently expanded to a sufficient scale to inject 25 5-6 week-old nude mice in the mammary fat pad with 2 x 10^6 cells. These mice will be treated in several groups with liposomal-DMSP which will consist of non-targeted SUVs composed of DMSP/DPPC/DSPC (1:1:1) at 4.0 mg or greater per dose, depending on the final outcome of the studies pertinent to Task 5. Single and multiple-dose regimens will be employed. Treatments will be initiated beginning in the first week after tumor implantation or delayed until tumors are ~5 mm in diameter.

Task 9

This Task will be undertaken once initial results from Task 8 are available, allowing comparisons of the anti-tumor efficacy of long-circulating, PEG-SUVs to those of the non-targeted SUVs.

Tasks 10 and 11

We still plan to undertake these after the corresponding mouse studies are well underway. It is possible that, in the unlikely event that we continue to experience difficulties with the mouse xenograft models with HER-2/neu over-expressing tumors, we will then switch to the rat breast adenocarcinoma model as the higher priority. We are clearly reluctant to do this, as this will not allow evaluation of the anti-tumor efficacy in a HER-2/neu over-expressing context, a major precept of the original proposal.

Tasks 12 and 13

These studies are certainly still planned, but have taken a lower priority than establishing the HER-2/neu tumor model for evaluation of anti-tumor efficay. They will be conducted with both non-targeted SUVs and PEG-SUVs.

Task 14

The most critical experiments to repeat will be those supporting the proof-of-principle, anti-tumor efficacy studies.

Key Research Accomplishments

Identified positive interaction between sphingolipid-induced apoptosis and anti-apoptotic role of Akt/PKB pathway in Her-2/neu-over-expressing human breast adenocarcinoma cells

Re-established human MDA-MB-361 HER-2/neu-over-expressing orthotopic human breast adenocarcinoma xenograft model in female nude mice

Reportable Outcomes

One manuscript, which was in preparation and considered nearly ready for submission, has been held up by us prior to submission to allow inclusion of both the studies in the MDA-MB-435/HER-2/neu transfectants model, as well as the molecular characterization of the Akt/PKB pathway with and without Wortmannin treatment. This will be the initial report on the effects of Her-2/neu expression on apoptotic responses to sphingolipids.

Conclusions

A major conclusion is that there is a positive interaction between sphingolipid-induced apoptosis and an anti-apoptotic role of the Akt/PKB pathway in Her-2/neu-over-expressing human breast adenocarcinoma cells. This can be at least partially overcome and thereby the breast tumor cell lines sensitized to sphingolipid-induced apoptosis by concomitant treatment with Wortmannin.

Another conclusion is more technical in nature and centers on the tumorigenicity of HER-2/neu-over-expressing MDA-MB-361 cells. Establishing this important model was a major and difficult undertaking, and we now believe that the culture conditions in vitro prior to implantation are very critical for successful implantation, at least at the orthotopic site involving the mammary fatpad. The culture conditions established/recommended by ATCC, including the use of non-bicarbonate/CO_2-based Liebowitz medium, and minimal expansion rates (1:2 or 1:3) upon passage to allow greater cell-to-cell communication, appear to be essential for preservation of morphology, avoidance of selection of variants, and optimal

implantation rates. It is regretable that re-establishing this model has taken the time and resources that we have experienced.

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